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Title: MULTI-ANALYSIS DETERMINATION OF TROPANE ALKALOIDS IN CEREALS AND SOLANACEAES SEEDS BY LIQUID CHROMATOGRAPHY COUPLED TO SINGLE STAGE EXACTIVE-ORBITRAP

Article Type: Full length article

Keywords: Tropane alkaloids; LC-MS-Orbitrap; Cereals; Solanaceaes seeds.

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Cover letter

UNIVERSIDAD DE ALMERÍA
Department of Chemistry and Physics

Almería, July 11, 2017

Dear Editor,

Please find enclosed the revised version of the manuscript former entitled "Multi-analysis determination of tropane alkaloids in cereals and solanaceaes seeds by liquid chromatography coupled to Exactive-Orbitrap", Manuscript Number JCA-17-935. The title has been modified to "Multi-analysis determination of tropane alkaloids in cereals and solanaceaes seeds by liquid chromatography coupled to single stage Exactive-Orbitrap", according to reviewers' suggestions.

We revised the manuscript taking into account the reviewers' comments (changes are indicated by using "Track Changes"). We have performed all the reviewers' suggestions, as it has been indicated in "*Response to Reviewers*". Moreover, the novelty of the work has been clarified and articulated, as well as more information regarding chromatographic separation has been included..

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I hope that the reviewing process finds the manuscript acceptable for publication in the journal.

Yours Sincerely,

Prof. Antonia Garrido Frenich

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Reviewers' comments:

Reviewer #1:

Comment: *The manuscript focuses on the establishment of a LC-MS platform used for quantifying tropane alkaloids in some plants. It is an important approach to monitor food safety and a systematic analysis work which was accomplished by some technologies. From the perspective of analytical areas, the work lacks novelty. So I am very sorry to say that it is not suitable for publication in the journal. I recommend the authors to submit it to food analysis research areas.*

Response: Up to now, most of the articles focused on the determination of tropane alkaloids in cereals and related products only determine atropine and scopolamine and there are scarce studies which include more compounds. Therefore EFSA (reference 2) is demanding multi tropane alkaloids methods. Only the recent EFSA study (reference 15, published in 2016) evaluates a high number of tropane alkaloids. However, several modifications have been performed in the current manuscript as the analysis of cuscohygrine, which was not included in EFSA report as well as the use of high resolution mass spectrometry. Moreover, a novel chromatographic method (coupling two columns) has also been proposed. In addition we tested the presence of TAs in contaminated samples in order to evaluate the potential contamination risks of cereals and related matrices with Datura stramonium and Brugmansia Arborea seeds. Thus the proposed manuscript is an interesting alternative to the one method published so far, increasing the number of possibilities for tropane alkaloids determination.

Comment: *The authors should provide a workflow which could help readers understand their works as soon as possible.*

Response: According to reviewer's indication, a workflow is included in the manuscript as figure (new Figure 2).

Comment: *Line 36, 98 "LC-MS- Orbitrap" is not a suitable abbreviation.*

Response: The abbreviation has been changed throughout the manuscript.

Comment: *In the method validation section, why not to validate the selectivity/specificity?*

Response: During method validation blank samples were injected in order to check selectivity, evaluating the presence of any signal at the same retention time of the target compounds. This has been indicated in the revised version of the manuscript.

Comment: *Line 271 In the paper, no detail information was provided on how to combine a Zorbax HILIC Plus with Eclipse Plus C18 column.*
Response: A serial column coupling was used, and further information regarding LC separation has been included in the revised version of the manuscript.

Comment: *The MS spectra profile of analytes should be provided in the paper.*
Response: The MS spectra had been included in the manuscript (Current Figure 3).

Comment: *In the reference section, the name of journal should not be in italic.*
Response: The references has been fixed up

Reviewer #2:

Comment: *The analysis of tropan alkaloids by HRMS is certainly an interesting topic. Yet the proposed methodology raises a number of questions.*

Chromatography: The authors report the poor chromatography (insufficient retention and bad peak shape) of many of their analytes. Their proposed combination of RP and HILIC is a very uncommon approach. Are these two columns connected? Which one is the first and which one the second? Based on the reported gradient. The elution is based on a HILIC regime (starting with ACN). Virtually all analytes elute within a narrow retention time window (6.5 to 8 minutes). It is unclear if not also the majority of the matrix compound elute within that time window. The authors do not present a chromatogram showing all the various tropan alkaloids. As mentioned, this kind of chromatographic separation is very uncommon. The authors certainly have to investigate this issue more deeply. Explaining the strengths and the limitation of this HILIC RP coupling.

Response: More information regarding serial column coupling has been added as well as a new figure (New Figure 4) showing the elution of two compounds with different stationary phases. The optimization of the separation of the target compounds was difficult bearing in mind the different physic-chemical characteristics of the target compounds. The use of a single column does not provide suitable results in terms of peak shape and retention time and that is why, two columns were connected, considering that HILIC and C18 stationary phases have orthogonal separation principles which could be useful to achieve a suitable separation of the target compounds.

Comment: *Signal suppression: Matrix effects are discussed following line 336. The authors considered only Soy as a potential problematic matrix. Indeed table 3 lists compounds like "Tropinone in soy" where values of " 0.002" are listed. This means that this compound gives a 500 times lower response in matrix than in the standard solution!? However, other matrices posed similar problems. It is really questionable if a quantitative method can be developed when suffering from such heavy matrix effects. This means that the proposed clean-up is insufficient and that the employed chromatography does not sufficiently resolve the analytes from the relevant matrix compounds.*

Response: Reviewer is right. The selected compounds have different physic-chemical properties and it is hard to find a common clean-up step to reduce matrix effect avoiding the loss of some the target analytes. Nevertheless it can be observed in Table 3 that despite the significant matrix effect, better LODs and LOQs were obtained when SPE was used.
Notwithstanding we are working in order to minimize this matrix effect, evaluating several alternatives.

Comment: *Line 23: It should be stated that this is a single stage orbitrap.*
Response: The correction has been included in the manuscript. Moreover, title has been modified according to this comment.

Comment: *Line 33/44: Too many values behind the decimal.*
Response: The decimal has been corrected in the manuscript and tables

Comment: *Line 76: SPE IS a clean-up technique.*
Response: This has been modified in the revised version of the manuscript.

Comment: *Line 81: Should this not read below pH of 7?*
Response: The mistake has been corrected.

Comment: *Line 140: Detection or separation?*
Response: It has been corrected.

Comment: *Table 1: The product ions are not clearly grouped for each analyte (table 1).*
Response: The table has been modified in order to avoid misunderstandings.

Reviewer #3:

Comment: *Although the English is generally good, the manuscript could be further revised in this sense to reduce some grammar erros.*

Response: English version of the manuscript has been revised.

Comment: *Line 140: Authors should mean separation instead of detection.*

Response: As it has been indicated above, this has been modified.

Comment: *Line 381: please, correct matriz.*

Response: The word has been corrected.

Highlights

- Simultaneous analysis of tropane alkaloids using LC-Oribtrap, coupling two LC columns
- Extraction of target compounds using SLE and SPE as clean-up and purification stage
- Determination of tropane alkaloids in buckwheat, millet, soy and linseed samples
- Contaminated buckwheat sample with *Datura Stramonium* and *Brugmansia Arborea* seeds

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2 **CEREALS AND SOLANACEAES SEEDS BY LIQUID CHROMATOGRAPHY**
3 **COUPLED TO SINGLE STAGE EXACTIVE-ORBITRAP**

4

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ABSTRACT

Tropane alkaloids are a wide group of substances that comprises more than 200 compounds occurring especially in the *Solanaceae* family. The main aim of this study is the development of a method for the analysis of the principal tropane alkaloids as atropine, scopolamine, anisodamine, tropane, tropine, littorine, homatropine, apoatropine, aposcopolamine, scopoline, tropinone, physoperuvine, pseudotropine and cuscohygrine in cereals and related matrices. For that, a simple solid-liquid extraction was optimized and a liquid chromatographic method coupled to a single stage Exactive-Orbitrap was developed. The method was validated obtaining recoveries in the range of 60-109% (except for some compounds in soy), precision values (expressed as relative standard deviation) lower than 20% and detection and quantification limits equal to or lower than 2 and 3 µg/kg respectively. Finally, the method was applied to the analysis of different types of samples as buckwheat, linseed, soy and millet, obtaining positives for anisodamine, scopolamine, atropine, littorine and tropinone in a millet flour sample above the quantification limits, whereas atropine and scopolamine were detected in a buckwheat sample, below the quantification limit. Contaminated samples with *Solanaceaes* seeds (*Datura Stramonium* and *Brugmansia Arborea*) were also analysed, obtaining concentrations up to 6943~~3.5~~ µg/kg (scopolamine) for contaminated samples with *Brugmansia* seeds and 18476~~.9~~ µg/kg (atropine) when samples were contaminated with *Stramonium* seeds.

Keywords: tropane alkaloids, LC-~~MS~~-Orbitrap~~-MS~~, cereals, *Solanaceaes* seeds

1. INTRODUCTION

Tropane alkaloids are large number of compounds which can be detected in numerous species of plants as *Erythroxylaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae* and *Cruciferae* [1], being more abundant in *Solanaceaes* plant family [2]. Although there are many cases of intended ingestions of parts of these plants for recreative [3] or medicinal use at low doses [4], normal ingestions occur when toxic plant parts (the highest concentration are usually detected in seeds [5]) are accidentally mixed with crops like grains, cereals, legumes and pseudocereals during harvest or processing [1,6], or ingested in herbal tea [7,8].

The most abundant tropane alkaloids are (-)-hyoscyamine, that racemizes to atropine under the extraction conditions used in pharmaceutical processes [2,9], and scopolamine. However ~~, although~~ there are more than 200 compounds present at lower concentrations [2,10,11], mainly produced by *Solanaceaes* plants (**Figure 1**) [12,13]. The principal studied compounds are atropine and scopolamine that have pharmacological effects because the inhibition of muscarinic acetylcholine receptors in the central nervous system (CNS) and the autonomic nervous system (ANS) [2]. They can produce dryness of the mucosa in the upper digestive and respiratory tract, pupil dilation (mydriasis), alterations in the heart rate, restlessness, irritability, ataxia, seizures, and respiratory depression [14].

Moreover, there are other tropane alkaloids (**Figure 1**) as anisodamine [11,12,15-18], which has the same effects but has less potency and toxicity than atropine and scopolamine, so it is more interesting for its use as a ~~medicine~~drug, littorine [12,15,18,19], homatropine [15,17,19,20], apoatropine [11,15], tropine [12,15,20] and tropinone [15] that were evaluated previously. However there are scarce studies [11,12,15,17-19] focused on the multi-analyte analysis of tropane alkaloids as it has

74 been required by European Food Safety Agency (EFSA) [2], and there are not any
75 method that enables the analysis of a large number of alkaloids in food and feed, where
76 tropane alkaloids can be found at concentrations higher than 100 µg/kg [2], except the
77 recent study requested by EFSA [15].

78 The extraction procedures will depend on the chemical properties of the compounds to
79 be analysed and specifically on the tropane alkaloids solubility [21] that is related to the
80 pH conditions, being more soluble in acid solutions [2]. The most common extraction
81 techniques are solid-liquid extraction (SLE), liquid-liquid extraction (LLE) or solid-
82 phase extraction (SPE). LLE was usually used to extract biological samples like plasma
83 and urine [19,22,23], being the samples mixed with acetonitrile for deproteinization and
84 an internal standard was utilized. The SPE extraction iswas used when the sample is
85 liquid (blood and urine), and the compounds retained in the SPE cartridge were eluted
86 with a mixture of ammonia (25%)/methanol (1:19, *v/v*) [24] or dichloromethane [25],
87 whereas for the extraction from plant material, compounds were eluted with 0.2%
88 trifluoroacetic acid (TFA), followed by 7.5 mL of methanol (0.2% in TFA):water (98:2,
89 *v/v*) [11]. Moreover, SPE is used during as clean-up stage technique [14,15]. SLE is the
90 most widely extraction technique used for plant material or matrices like buckwheat.
91 Due to the polar nature of tropane alkaloids, the target compounds were extracted using
92 alcoholic solutions [26,27], mixtures of ethanol or methanol and water [8,15] or
93 mixtures of water and acetonitrile [15,20]. In general, when these mixtures were used,
94 the pH of the water is ≥≤ 7, since most of the tropane alkaloids, except apoatropine,
95 cocaine and the truxillines, are fairly water soluble at acidic pH [21].

96 The chromatographic analysis can be performed by electrophoretic techniques [28] or
97 gas chromatography [21,28-30], although liquid chromatography (LC) is the most
98 widely used [15,19,26]. Triple quadrupole (QqQ) is mostly utilized [12,17], because it

provides lower detection and quantification limits than other analysers, which were less frequently used as time of flight-mass spectrometry (TOF-MS) [31], quadrupole-time of flight (QTOF) [10] or quadrupole-Orbitrap (Q-Orbitrap) [20,32]. These high resolution mass spectrometry (HRMS) analysers are commonly used in profiling studies of *Solanaceaes* species, although they were also used for the multi-analysis of a huge number of toxins [33] and some of ~~this~~ them ~~including~~ included few tropane alkaloids [20]. Several parameters such as retention time windows (RTW), accurate mass error (<5ppm) and isotopic pattern can be used when HRMS is utilized for the identification of target compounds. Furthermore, they can provide the identification of unknown compounds [34].

Due to there are ~~few~~ scarce studies focused on ~~-~~multi-analyte tropane alkaloids determination, the objective of this manuscript is the development of a new method by LC-~~MS~~-Orbitrap MS that allows for the determination of different tropane alkaloids present naturally in *Solanaceaes* seeds. ~~and, t~~Therefore, they ~~can~~ could be detected in cereals and oilseeds, fulfilling EFSA requirements regarding the determination of multi tropane alkaloids in several matrices ~~studying most of the tropane alkaloids indicated by EFSA~~ [2]. In addition, the developed method has been applied to the analysis of samples belonging to the principal seeds matrices that can be contaminated as buckwheat, linseed, soy and millet as well as intentionally contaminated matrices with *Datura Stramonium* and *Brugmansia Arborea* seeds were also determined.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Scopolamine, atropine, anisodamine, tropane, tropine, homatropine and tropinone reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA);

124 littorine, apoatropine, cuscohygrine and aposcopolamine reference standards were
125 obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and scopoline,
126 physoperuvine and pseudotropine were purchased from Chemfaces (Wuhan, China). All
127 compounds present a purity ≥ 98% except tropine (≥ 97%).

128 Stock standard solutions of the compounds (200 mg/L) were individually prepared by
129 exact weighing of the solid substances and dissolved in 50 mL of methanol (~~HPLC~~ LC-
130 MS grade, J.T. Baker, Deventer, ~~Holland~~The Netherlands), and they were stored at -20
131 °C in the dark. Then a mixture working standard solution was prepared at 5 mg/L with
132 methanol and stored in screw-capped glass tubes at -20 °C in the dark. The stock
133 standard solutions were stable up to one year, whereas working standard solutions were
134 prepared every two months.

135 LC-MS grade acetonitrile was obtained from VWR Chemicals (Radnor, PE, USA)
136 and water from J.T. Baker. Formic acid (Optima LC-MS) was supplied from Fisher
137 Scientific (Geel, Belgium) while ammonium hydroxide 25% solution was obtained from
138 Fluka (Steinheim, Suiza). Graphitized black carbon (GBC) and primary secondary
139 amine (PSA) were purchased from Scharlab (Barcelona, Spain). Anhydrous magnesium
140 sulphate, ammonium acetate and acetic acid were purchased from Panreac (Barcelona,
141 Spain).

142 For the SPE preconcentration and clean-up stage OASIS MCX 150 mg/6 mL (Waters,
143 Milford, MA, USA) and a Strata-X-C 200 mg/3 mL (Phenomenex, Torrance, CA, USA)
144 cartridges were used.

145 A 0.22 µm nylon syringe filters provided by Agilent Technologies (Santa Clara, CA,
146 USA) were used.

147

148 *2.2. Instrument and apparatus*

149 The samples were ground into powder using an automatic blender (Sammic S.L., 150 Azkoitia, Spain). To homogenize the samples, a WX vortex from Velp Scientifica 151 (Usmate, Italy) was used and for the extraction, a Reax 2 rotatory shaker from Heidolph 152 (Schwabach, Germany) was utilized. A Consul 21 centrifuge from Orto Alresa (Madrid, 153 Spain) was used for centrifugation.

154 SeparationDetection of the compounds was performed with a Thermo Fisher 155 Scientific Transcend 600 LC (Thermo Scientific Transcend^{TM}, Thermo Fisher 156 Scientific, San Jose, CA, USA).

157 A Zorbax Eclipse Plus C_{18} column (100 × 2.1 mm, 1.8 µm particle size) and a Zorbax 158 Eclipse Plus HILIC (100 × 2.1 mm, 3.5 µm particle size) were used for separation 159 (Agilent Technologies).

160 A mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive^{TM}, Thermo Fisher 161 Scientific, Bremen, Germany) with electrospray interface (ESI) (HESI-II, Thermo 162 Fisher Scientific, San Jose, CA, USA) in positive mode was used for the detection, 163 identification and quantification purposes. The following parameters were applied: 164 spray voltage, 4 kV; sheath gas (N_2, > 95%), 35 (adimensional); auxiliary gas (N_2, > 165 95%), 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens 166 voltage, 95 V; heater temperature, 305 °C; capillary temperature, 300 °C.

167 For the mass spectra acquisition, first a full-scan MS, (ESI^+), without fragmentation 168 (higher collisional dissociation (HCD) collision cell was switched off), at mass 169 resolving power = 25,000 FWHM, was used to obtain the molecular ion of each 170 compound. Then, fragments were obtained using all-ion fragmentation (AIF) mode, 171 ESI^+, with fragmentation (HCD on, collision energy = 30 eV), at mass resolving power 172 = 10,000 FWHM.

173 Finally, the chromatograms were processed using Xcalibur™ version 2.2, with
174 Quanbrowser and Qualbrowser, and Mass Frontier™ 6.0 (Thermo Fisher Scientific, Les
175 Ulis, France).

176

177 *2.3. Samples collection*

178 Samples were obtained from local supermarkets located in Almería (Spain). The
179 analysed samples were buckwheat, buckwheat flour and buckwheat pasta (*Fagopyrum*
180 *esculentum)*, soy and soy flour (*Glycine max)*, peeled millet and millet flour *(Panicum*
181 *miliaceum)* and linseed and linseed flour (*Linum usitatissimum*), which were selected
182 according to EFSA`’s concern regarding food control of tropane alkaloids [2] and
183 previous studies [35]. Before extraction, samples were ground into powder. Blank
184 samples were used for the preparation of fortified samples.

185 Two *Solanaceaes* seeds, *Datura Stramonium* [30] and *Brugmansia arborea* [36] plant
186 seeds, used to contaminate cereal samples, were collected from Huelva (Spain). For the
187 contamination of the samples, two methods were applied: 1 g of *Stramonium* and
188 *Brugmansia* seeds was mixed with 1 kg of sample, bearing in mind previous studies
189 [30,35], and 0.1 g for *Stramonium* seeds and 0.5 g for *Brugmansia* seeds were mixed
190 with 1 kg of sample (approximately 1 seed of each plant in 100 g of sample). The
191 mixture was homogenized during 2 hours in a rotatory agitator before aliquots were
192 collected to perform the extraction of the target compounds.

193

194 *2.4. Sample preparation*

195 A simple solid-liquid extraction procedure, based on previous method [8], was
196 employed, showing a scheme of the procedure in **Figure 2**. Briefly, one gram of sample
197 was mixed with 10 mL of a solution containing water (0.5% acetic acid):methanol (1:2

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198 *v/v*) for 1 minute in a vortex. Then, it was mixed for 30 minutes in a rotatory agitator
199 and after that, the mixture was centrifuged at 5000 rpm for 10 min. The supernatant was
200 passed through a strong cationic exchange SPE cartridge, previously conditioned with
201 methanol and methanol:water 1% acetic acid (2:1 *v/v*), washed with methanol:water 1%
202 acetic acid (2:1 *v/v*) and the compounds were eluted with methanol containing 3% of
203 ammonium hydroxide solution (25%). The extract was evaporated under nitrogen flow
204 and reconstituted in 500 µL of methanol:water 0.1% formic acid (50:50 *v/v*). Finally,
205 the sample was filtered into a 0.22 µm nylon syringe filter and injected into the LC
206 system.

207

208 *2.5. LC-Orbitrap MS analyses*

209 The chromatographic separation was carried out employing a binary mobile phase
210 with acetonitrile (A) and an aqueous solution of formic acid (0.1%, *v/v*) (B) at flow rate
211 of 0.2 mL/min. The gradient elution started at 90% of A and held 1 min ~~at flow rate of~~
212 ~~0.2 mL/min~~. This composition was reduced to 70% A in 4 min, and then to 50% A in 2
213 min and finally to 5% A at 9 min. This composition was held for 1 min. The
214 composition was increased to 50% A in 2 min and was returned to the initial conditions
215 in 2 min, equilibrating the stationary phase during 4 min. The total running time was 18
216 min. Injection volume was 10 µL and column temperature was kept at 25°C (room
217 temperature).

218 The compounds were ionized with electrospray ionization (ESI) in positive mode.
219 Retention time and MS/MS parameters are shown in **Table 1**.

220

221 *2.6. Method validation*

222 The method was validated following the indications of the SANTE guidelines [37]
223 and the AOAC Guidelines for Dietary Supplements and Botanicals [38]. Parameters
224 evaluated were: linearity, matrix effect, selectivity, trueness in term of recoveries,
225 precision intra and interday and the detection (LOD), identification (LOI) and
226 quantification (LOQ) limits.

227 Linearity was appraised with a matrix-matched calibration, spiking blank extracts
228 samples at the concentration of 0, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/kg.

229 To evaluate the matrix effect, matrix-matched calibrations of the different matrices
230 (buckwheat, millet soy and linseed) and in solvent were prepared at concentration
231 between 0.5-100 µg/kg and compared.

232 Selectivity was evaluated by the analysis of blank samples.

233 Trueness was investigated through recovery trials, spiking blank samples at three
234 levels (1, 5 and 50 µg/kg) for buckwheat, millet, soy and linseed. Each fortified
235 concentration was repeated five times.

236 Precision was performed in terms of repeatability (intra-day precision) and
237 reproducibility (inter-day precision), expressed as relative standard deviation (RSD, %).
238 For intra-day precision, five replicates at three concentration levels (1, 5 and 50 µg/kg)
239 were evaluated. For inter-day precision, spiked blank samples were tested at the same
240 concentration of the intra-day precision for buckwheat and at the concentration of 5
241 µg/kg for the other matrices, during 5 days.

242 Finally for the estimation of the LOD, LOI and LOQ, extracted blank samples before
243 and after the SPE process were spiked at low concentrations between 0.1 to 25 µg/kg.
244 The LOD was defined as the minimum concentration at which the characteristic ion is
245 monitored with a mass error lower than 5 ppm; the LOI as the minimum concentration
246 at which the characteristic ion and one fragment at least is monitored with a mass error

lower than 5 and 10 ppm respectively, and the LOQ as the minimum concentration at which the characteristic ion and one fragment at least is monitored with a mass error lower than 5 and 10 ppm respectively, providing good linearity and recoveries.

3. Results and discussion

3.1. Optimization of LC-Orbitrap-MS

First the MS conditions were optimized. Full-scan MS (ESI+) was applied to obtain the characteristic ion for each compound. For this purpose, the target compounds individually prepared at 1 mg/L were injected into the LC-Orbitrap system. The characteristic ions were in every case the protonated molecular ion (**Figure 3~~2~~**). Then, fragments were obtained using all-ion fragmentation (AIF) in positive mode, setting the collision energy at 30 eV. The fragments obtained were picked out according to their sensitivity and their formulas were proposed by fragmentation of its weakest links, using the Massfrontier software in some cases. Then the fragments were proposed according to the parent structure of the compound (**Figure 3~~2~~**).

The most frequent and common break of tropane alkaloids is produced by the oxygen of the ester of the structure, leaving only the tropane ring. Thus, and depending of the atoms tied to the tropane ring, the mass of the product ions obtained could be *m/z* 124.1121 (if nothing else is connected with the ring as atropine, littorine, homatropine, apoatropine and pseudotropine), *m/z* 138.0913 (when an epoxy ring is connected; scopolamine and aposcopolamine) or *m/z* 140.1070 (if there is a hydroxyl group; anisodamine). Other common fragmentations are the oxygen of the ester and the nitrogen of the tropane ring, that generates the fragment *m/z* 93.0699 (atropine, littorine, homatropine, apoatropine, tropine, physoperuvine and pseudotropine), the link of the ester with the α-carbon, that provides the fragment *m/z* 103.0542 (scopolamine,

aposcopolamine and apoatropine) and finally the ester link, that gives the fragment *m/z* 156.1019 (scopolamine) or *m/z* 142.1226 (littorine and homatropine), (see **Table 1** and **Figure** 3~~2~~). For compounds with the same characteristic ion, as atropine and littorine, which also have similar retention time (6.69-6.90 and 6.60-6.81 minutes respectively) this fragment (*m/z* 142.1226) allow their quantification separately.

Then different columns were tested: Hypersil Gold aQ (100x2.1 mm, 1.9µm) (Thermo Fisher Scientific, Bremen, Germany), Zorbax Eclipse Plus C8 (100x2.1 mm, 1.8µm) (Agilent Technologies), Hypersil Gold Phenyl (100x2.1 mm, 1.9µm) (Thermo Fisher Scientific, Bremen, Germany), Zorbax Hilic plus (100x2.1 mm, 3.5 µm) (Agilent Technologies) and Zorbax Eclipse Plus C18 (100x2.1 mm, 1.8µm) (Agilent Technologies). When these columns were used, the separation is good for high molecular weight compounds but for the low molecular weight substances (tropine, pseudotropine, cuscohygrine, physoperuvine and tropinone) the separation is bad and the retention time is too short (minute 1). This could make that the compounds eluted next to the compounds present in the matrices and strong matrix effect could be presented. For this reason, ~~the combination~~a serial column coupling of ~~two columns, ~~a Zorbax HILIC Plus and Eclipse Plus C_{18} column was used, coupling HILIC ~~column~~stationary phase (first column) with~~ after~~ C_{18} (second column)~~, obtaining the best peak forms and suitable retention times, ranging from 6.26 (apoatropine) to 11.49 (cuscohygrine) min~~. The compounds are retained in the HILIC column until the minute 3 and then they are retained in the C_{18} column, obtaining the best peak ~~forms~~shape and suitable retention times, ranging from 6.26 (apoatropine) to 11.49 (cuscohygrine) min, ~~apart ~~as well as ~~from a~~a better sensitivity. As example ~~(~~**Figure 4** shows the extracted ion chromatograms of tropane and anisodamine when the separation was performed

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296 using only one column (C18 or HILIC) or when both columns were coupled, which

297 provides better results (suitable elution time and peak shape)).

298

299 *3.2. Optimization of the extraction procedure*

300 The first extraction procedure tested was a modified QuEChERS procedure previously

301 developed [35], where the phase separation of water and acidified acetonitrile permits

302 the extraction of atropine and scopolamine. While the recoveries obtained for the high

303 molecular weight compounds were acceptable, for the low molecular weight and polar

304 compounds, recoveries were lower than 40% (**Table 2**). Other extraction procedures

305 based on the original QuEChERS methodology were checked. QuEChERS

306 methodology with acetonitrile containing 1% formic acid and water without phases

307 separation (no salts were added) was tested in order to avoid that the polar analytes kept

308 in the aqueous phase, and the QuEChERS methodology with different percent of

309 water:acidified acetonitrile were checked, but the recoveries were lower than 60% for

310 most of the target compounds in both cases.

311 It is well-known that the majority of tropane alkaloids are very soluble in acid

312 aqueous solutions, especially low molecular weight compounds [21]. For this reason a

313 possible strategy to get the complete extraction of the target analytes is the use of

314 basified aqueous solutions for the migration of the compounds to the organic phase.

315 Thus, a modification of the original method employed in a previous study [35] was

316 evaluated, employing testing water containing 2% ammonium hydroxide solution (25%)

317 and acetonitrile. The recoveries of the low molecular weight compounds improved (20-

318 48%) but they decreased for high molecular weight compounds (**Table 2**), being lower

319 than 63%. This can be explained considering that compounds can be extracted from the

solid sample to the acidified aqueous phase, whereas if basified aqueous phase was used, the compounds were kept in the sample.

Moreover, the extraction without water was tested, employing 10 mL of methanol containing 0.5% acetic acid (**Table 2**), but the recoveries remained low (23-70 %), because the compounds are more soluble in acidified aqueous medium than in acidified methanol.

Knowing that the compounds were kept in the aqueous phase, an extraction procedure developed by Shimshoni et al (2015) [8], was employed, using a mixture of methanol:water 0.5% acetic acid (2:1 *v/v*). Following this methodology the recoveries improved (79-103%) (**Table 2**) but the dilution factor of the method provoked that the LOD and LOQ values were higher than those recommended by EFSA [15]. In order to improve the lower limits of the method, a mixture of methanol:water 0.5% acetic acid (90:10 *v/v*) or methanol 0.5% acetic acid were tested, and 10 mL of extract were evaporated and redissolved in 500 µL of mobile phase, but the recoveries are lower than 70% in every case.

A~~n extra step of~~ preconcentration and clean-up stage with a SPE method could be applied using strong cation exchange (SCE) cartridges like Oasis MCX [15,39,40], StrataX [15], SCX [14] or Cleanert PCX [24]. The Oasis MCX and StrataX were tested, obtaining better result with the StrataX cartridges. For low molecular weight compounds, recovery ranged from 50-80% when Oasis MCX cartridges were used, whereas if Strata X cartridges were utilized recovery ranged from 75 to 93%. The conditioning of the cartridge was performed with methanol and methanol:water from 0% to 1% acetic acid (2:1 *v/v*), providing better results when methanol:water 1% acetic acid (2:1 *v/v*) was used. The washing of the cartridges was performed with the same mixture used for the conditioning step. The cartridges were dried 30, 60 and 120

345 minutes under vacuum stream, choosing 1 h60 min. Finally the elution of the
346 compounds was tested utilizing methanol with different percentages of ammonium
347 hydroxide solution (25%) from 0.5 to 3%, or methanol containing sodium hydroxide
348 (0.05 M). The best result (recoveries between 68-93%) were obtained when methanol
349 containing 3% of the ammonium hydroxide solution (25%) was used.

350 After the nitrogen flow evaporation, the extracts were recomposed with different
351 mixtures of methanol:water: 100:0 (*v/v*), 50:50 (*v/v*), 50:50 with 0.1% formic acid (*v/v*),
352 10:90 (*v/v*) and 10:90 with 0.1% formic acid (*v/v*), providing the best results when the
353 mixture of methanol:water containing 0.1% formic acid (50:50 *v/v*) was used, obtaining
354 recoveries ranging from 63-109%. A workflow of the method is shown in the **Figure 2**.

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356 *3.3. Method validation*

357 Linearity (**Table 3**) was evaluated through coefficients of determination (R^2), which
358 were higher than 0.993, as well as residuals, which were always lower than 20%. Also,
359 **Table 3** shows that there is a high significant difference between the slope obtained in
360 solvent and in the tested matrix. It can be seen that the matrix effect was similar for the
361 majority of compounds in the tested samples except in soy (strong matrix suppression),
362 so it is possible the use of a representative matrix for quantification purposes except for
363 soy.

364 The selectivity of the method was evaluated by extracting and analyzing blank
365 samples. The absence of any chromatographic signal when the characteristic ion of each
366 compound was monitored at the same retention time of the selected compounds
367 indicated the absence of chemical or matrix interferences.

368

—Recoveries ranged from 60-109% except for soy at 1 μg/kg for tropine, pseudotropine and tropane (34-53%), as it can be observed in **Table 4**.

The precision was evaluated as intra and interday precision (**Table 4**), expressed in terms of relative standard deviation (RSD) and good values were obtained, ranging from 1 to 20%. Although some recoveries were lower than 60%, a correction factor could be used for the quantification of these compounds in this matrix considering the repeatability of the proposed method.

Lower limits were calculated and they range from 0.1 to 2 μg/kg (LOD) and from 0.5 to 3 μg/kg (LOI and LOQ), despite the matrix suppression obtained for all the compounds in the evaluated matrices. For instance, ~~. S~~some compounds present ~~a~~ lower LOQs in millet than in buckwheat, due to its lower matrix effect. Applying the developed methodology, similar limits than the recent study developed by EFSA [15] were obtained for a large group of tropane alkaloids. It is important to highlight that there are not legal limits for these compounds in food and feed, except the recent normative of the European Union about atropine and scopolamine in cereal based products for children, which set a maximum allowable content of 1 μg/kg for atropine and scopolamine [41].

It can also be observed that without the application of the SPE, for some compounds as atropine, scopolamine, anisodamine and tropane, the limits were only slightly higher than those obtained with the SPE process, whereas the other compounds had higher limits without the SPE (5-25 μg/kg) (**Table 3**). ~~However~~Thus, if low limits are not required, the method can be applied without SPE process, simplifying the analytical procedure in routine analysis.

3.4. Real samples analysis

Samples (15 in total) analysed were soy, linseed and millet, seeds and flour, and buckwheat seeds, flour and pasta. Target compounds were only detected in buckwheat and millet flour (**Figure** 5~~3~~). For the buckwheat sample, atropine and scopolamine were detected but at concentrations below the LOQ and for the millet flour sample the compounds detected were anisodamine, scopolamine, atropine, littorine and tropinone, being the highest concentration of 23~~.4~~ µg/kg for scopolamine (**Table 5**).

In addition, contaminated samples with *Datura Stramonium* and *Brugmansia Arborea* according to previous studies [9,30] were evaluated (see section *2.3. Sample collection*). The concentration obtained (**Table 5**) shown that the largest concentrations were different for both seeds. For *Brugmansia* seeds, the highest concentrations were obtained for anisodamine (115~~4.6~~ µg/kg), atropine (292~~1.9~~ µg/kg), scopolamine (693~~.5~~ µg/kg) and homatropine (337~~.4~~ µg/kg) and all the compounds included in this study were detected except aposcopolamine, apoatropine, cuscohygrine, tropinone and tropane. When samples were contaminated with *Stramonium* seeds, the compounds detected at the highest concentrations were scopolamine (~~89.7~~90 µg/kg), littorine (139~~.0~~ µg/kg) and atropine (1847~~6.9~~ µg/kg), whereas aposcopolamine, cuscohygrine and tropane were not detected. The highest concentration was obtained for scopolamine at the samples contaminated with *Brugmansia* and atropine (much higher than the rest of compounds) for *Stramonium*. ~~Even with~~Despite the small amount of *Solanaceaes* seeds plant added (10 and 50 mg per 100 g matrix~~z~~ ≈ 1 seeds of *Datura Stramonium* and *Brugmansia Arborea* respectively) the concentration in tropane alkaloids was higher than 100 µg/kg for atropine (*Stramonium* and *Brugmansia*), scopolamine and homatropine (*Brugmansia*), which exceed the limits set by European Union [41].

4. Conclusions

The proposed method provides a rapid and simple determination of a large number of tropane alkaloids present in ~~the~~ *Solenaceaes* plants, and it can be used to evaluate the possible contamination of different matrices with these compounds. For this, a solid-liquid extraction with a SPE purification step process and the LC-Orbitrap -MS analysis were used. The validation parameters were suitable for the compounds and matrices included in this study, except the recoveries obtained for some compounds in soy at low concentrations. The LOD and LOQ values allow for the determination at very low concentration of tropane alkaloids in real samples. A total of 15 real samples were analyzed using the developed method, detecting other tropane alkaloids in addition of atropine and scopolamine as anisodamine, littorine and tropinone. Finally the contaminated samples shown that the concentration obtained with a low quantity of *Solanaceaes* seeds added were higher than the established limits.

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Figure captions

Figure 1: Scheme of tropane alkaloid biosynthesis pathways (Adapted from references 12 and 13).

Figure 2: ~~Compound analysis w~~Workflow of the proposed analytical method. ~~Characteristic and fragment ions mass spectrum of each target compound.~~

Figure 3: Characteristic and fragment ions mass spectrum of each target compound. ~~Chromatogram and mass spectrum of the positive millet flour sample: A) Extracted ion chromatogram of scopolamine (23.4 µg/kg); B) Extracted fragment ion chromatogram at *m/z* 156.1022; C) Experimental mass spectrum of scopolamine, and D) Theoretical mass spectrum of scopolamine~~

Figure 4: Effect of single and serial column coupling on the separation of A) tropane and B) anisodamine.

Figure 5: Chromatogram and mass spectrum of the positive millet flour sample: A) Extracted ion chromatogram of scopolamine (23~~.4~~ µg/kg); B) Extracted fragment ion chromatogram at *m/z* 156.1022; C) Experimental mass spectrum of scopolamine, and D) Theoretical mass spectrum of scopolamine.

Table 1: Retention time windows (RTWs) and MS/MS parameters for the target compounds

Compound	RTW (min)	Precursor ion		Fragments		
		Exact mass	Mass error (ppm)	Exact mass	Molecular formula	Mass error (ppm)
Anisodamine	6.58-6.96	306.1699	2.66	140.1070	$C_8H_{14}NO$	-0.93
				91.0542	C_7H_7	4.54
				122.0964	$C_8H_{12}N$	-4.64
Scopolamine	6.75-6.98	304.1543	3.83	138.0913	$C_8H_{12}NO$	4.84
				121.0648	C_8H_9O	4.61
				156.1019	$C_8H_{14}NO_2$	1.88
				103.0542	C_8H_7	4.98
Atropine	6.69-6.90	290.1751	3.21	124.1121	$C_8H_{14}N$	4.46
				93.0699	C_7H_9	3.36
Littorine	6.60-6.81	290.1751	4.58	124.1121	$C_8H_{14}N$	3.42
				142.1226	$C_8H_{16}NO$	3.09
				93.0699	C_7H_9	3.79
Aposcopolamine	6.28-6.61	286.1438	-1.30	103.0542	C_8H_7	4.20
				138.0913	$C_8H_{12}NO$	-2.90
Homatropine	6.68-6.88	276.1594	4.27	124.1121	$C_8H_{14}N$	4.95
				142.1226	$C_8H_{16}NO$	2.47
				93.0699	C_7H_9	2.72
Apoatropine	6.26-6.60	272.1645	-1.86	124.1121	$C_8H_{14}N$	2.85
				93.0699	C_7H_9	3.15
				103.0542	C_8H_7	4.59
Cuscohygrine	11.31-11.49	225.1961	-1.95	84.0808	$C_5H_{10}N$	4.09
Scopoline	7.84-7.98	156.1019	-0.03	138.0913	$C_8H_{12}NO$	0.79
				96.0808	$C_6H_{10}N$	0.04
Tropine/ Physoperuvine	7.83-8.10	142.1226	2.53	98.0964	$C_6H_{12}N$	4.73
				96.0808	$C_6H_{10}N$	3.27
				93.0699	C_7H_9	4.65
Pseudotropine	7.85-8.13	142.1226	-0.99	96.0808	$C_6H_{10}N$	4.83
				124.1121	$C_8H_{14}N$	2.85
				93.0699	C_7H_7	1.43
Tropinone	7.56-7.95	140.1070	2.92	98.0964	$C_6H_{12}N$	-0.87
				57.0573	C_3H_7N	3.66
Tropane	7.72-7.86	126.1277	3.76	95.0855	C_7H_{11}	3.08
				70.0651	C_4H_8N	0.06

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Table 2: Recoveries obtained for the different extraction procedures evaluated from spiked samples at 100 µg/kg

Compounds	Recoveries[a,b]			
	Extraction 1	Extraction 2	Extraction 3	Extraction 4
Anisodamine	79 (10)	62 (12)	69 (3)	95 (3)
Scopolamine	72 (13)	53 (11)	70 (4)	94 (4)
Atropine	73 (7)	51 (10)	62 (5)	91 (2)
Littorine	70 (2)	52 (5)	63 (3)	103 (1)
Aposcopolamine	87 (1)	61 (6)	48 (3)	95 (4)
Homatropine	66 (11)	50 (15)	62 (2)	93 (2)
Apoatropine	93 (2)	63 (3)	55 (1)	99 (1)
Scopoline	19 (4)	30 (6)	23 (1)	93 (6)
Cuscohygrine	17 (11)	20 (18)	39 (11)	93 (14)
Tropine/Physoperuvine	24 (3)	29 (3)	38 (6)	91 (2)
Pseudotropine	23 (13)	25 (10)	26 (5)	89 (6)
Tropinone	40 (3)	48 (5)	57 (3)	91 (4)
Tropane	19 (14)	25 (11)	58 (15)	95 (4)

[a] Extraction 1= QuEChERS extraction; Extraction 2 = Basified QuEChERS; Extraction 3 = Methanol 0.5% acetic acid extraction; Extraction 4= Methanol:water 0.5% acetic acid (2:1 *v/v*)
[b] Repeatability is given in brackets (n=5)

Table 3: Linearity, matrix effect and lower limits for the target tropane alkaloids in cereal samples

Compounds	Linearity (R^2)	Matrix effect with SPE purification[a]				Limits with SPE purification (μg/kg)			Limits without SPE purification (μg/kg)		
		Buckwheat	Millet	Soy	Linseed	LOD	LOI	LOQ	LOD	LOI	LOQ
Anisodamine	0.997	0.332	0.635	0.431	0.543	0.5	0.5	1	1	2	2
Scopolamine	0.993	0.198	0.303	0.352	0.334	1	1	2	2	3	5
Atropine	0.997	0.315	0.375	0.351	0.338	0.5	1	1	1	2	2
Littorine	0.996	0.289	0.391	0.361	0.345	0.5	1	2	5	5	10
Aposcopolamine	0.996	0.174	0.203	0.271	0.131	1	1	2	2	2	5
Homatropine	0.997	0.218	0.328	0.330	0.283	0.1	0.5	0.5	2	5	5
Apoatropine	0.997	0.212	0.465	0.384	0.213	1	1	2	2	2	5
Scopoline	0.996	0.029	0.091	0.054	0.013	1	1	2	10	10	25
Cuscohygrine	0.995	0.592	0.412	0.396	0.521	2	3	3	5	5	10
Tropine/Physoperuvine	0.996	0.031	0.062	0.005	0.012	0.5	0.5	1	10	10	25
Pseudotropine	0.996	0.042	0.078	0.011	0.030	0.5	0.5	1	10	10	25
Tropinone	0.996	0.044	0.067	0.002	0.013	0.5	1	2	10	10	25
Tropane	0.995	0.024	0.032	0.005	0.013	0.1	0.5	0.5	1	1	2

[a] Calculated as the quotient between the matrix and solvent slopes

Table 4: Validation parameters of the optimized method

Compounds	Matrix	Recoveries[a]			Interday precision (n=5)		
		1 µg/kg	5 µg/kg	50 µg/kg	1 µg/kg	5 µg/kg	50 µg/kg
Anisodamine	Buckwheat	63 (10)	84 (7)	91 (2)	11	6	1
	Millet	72 (8)	90 (5)	93 (4)	-	5	-
	Soy	66 (11)	84 (9)	81 (3)	-	8	-
	Linseed	67 (12)	71 (8)	75 (3)	-	9	-
Scopolamine	Buckwheat	-	94 (5)	86 (4)	-	6	8
	Millet	63 (9)	91 (3)	90 (3)	-	5	-
	Soy	-	91 (8)	84 (6)	-	8	-
	Linseed	-	72 (8)	83 (5)	-	6	-
Atropine	Buckwheat	65 (10)	91 (7)	91 (4)	13	10	5
	Millet	76 (5)	93 (6)	92 (4)	-	7	-
	Soy	63 (11)	90 (13)	86 (6)	-	11	-
	Linseed	63 (13)	79 (9)	83 (3)	-	10	-
Littorine	Buckwheat	-	75 (7)	80 (1)	-	10	2
	Millet	74 (11)	87 (6)	88 (1)	-	4	-
	Soy	-	81 (11)	79 (8)	-	9	-
	Linseed	-	65 (8)	84 (3)	-	6	-
Aposcopolamine	Buckwheat	-	74 (6)	75 (6)	-	15	7
	Millet	70 (15)	92 (7)	97 (6)	-	11	-
	Soy	-	60 (7)	78 (8)	-	9	-
	Linseed	-	75 (10)	83 (9)	-	10	-
Homatropine	Buckwheat	66 (8)	86 (6)	93 (1)	13	12	2
	Millet	81 (8)	94 (5)	89 (3)	-	8	-
	Soy	70 (9)	84 (10)	86 (2)	-	10	-
	Linseed	68 (11)	72 (5)	81 (6)	-	12	-
Apoatropine	Buckwheat	-	60 (5)	68 (2)	-	6	3
	Millet	68 (12)	73 (6)	82 (3)	-	10	-
	Soy	-	60 (8)	69 (5)	-	7	-
	Linseed	-	61 (8)	69 (3)	-	11	-
Scopoline	Buckwheat	-	67 (14)	81 (8)	17	11	13
	Millet	64 (19)	74 (5)	89 (3)	-	9	-
	Soy	-	69 (16)	90 (8)	-	17	-
	Linseed	-	83 (14)	91 (9)	-	7	-
Cuscohygrine	Buckwheat	-	66 (9)	76 (8)	-	14	4
	Millet	-	71 (10)	89 (5)	-	14	-
	Soy	-	61 (13)	82 (11)	-	14	-
	Linseed	-	62 (12)	79 (8)	-	15	-
Tropine/ physoperuvine	Buckwheat	67 (13)	84 (11)	80 (2)	16	14	6
	Millet	72 (8)	90 (8)	85 (5)	-	10	-
	Soy	34 (15)	95 (12)	109 (4)	-	15	-
	Linseed	63 (12)	81 (11)	82 (3)	-	16	-
Pseudotropine	Buckwheat	74 (19)	84 (16)	87 (9)	15	16	5
	Millet	66 (12)	97 (9)	91 (6)	-	17	-
	Soy	53 (13)	95 (15)	101 (11)	-	14	-
	Linseed	73 (11)	79 (12)	85 (9)	-	11	-
Tropinone	Buckwheat	-	79 (19)	88 (3)	-	20	7
	Millet	-	70 (11)	92 (5)	-	16	-
	Soy	-	62 (20)	97 (6)	-	20	-

	Linseed	-	74 (13)	88 (5)	-	13	-
Tropane	Buckwheat	63 (20)	64 (16)	88 (9)	15	17	9
	Millet	63 (18)	69 (15)	81 (4)	-	13	-
	Soy	47 (17)	60 (16)	69 (11)	-	18	-
	Linseed	63 (16)	68 (17)	72 (10)	-	13	-

[a] Intraday precision is given in brackets (n=5)

Table 5: Concentrations (µg/kg) of detected compounds in the contaminated samples

Compounds	Concentration (µg/kg)[a]				Millet flour positive sample
	Brugmansia contamination		*Stramonium* contamination		
	1 g/kg[b]	0.5 g/kg	1 g/kg	0.1 g/kg	
Anisodamine	115~~4.6~~ (13)	46~~5.6~~ (10)	23~~2.9~~ (6)	3~~2.6~~ (8)	5~~.4~~ (4)
Scopolamine	694~~3.5~~ (12)	277~~.2~~ (6)	90~~89.7~~ (4)	6~~.4~~ (11)	23~~.4~~ (6)
Atropine	292~~1.9~~ (7)	142~~.4~~ (8)	184 7~~6.9~~ (1)	169~~8.5~~ (6)	13~~12.7~~ (6)
Littorine	16~~.4~~ (9)	5~~4.5~~ (12)	139~~.0~~ (3)	13~~.2~~ (13)	3~~2.8~~ (19)
Homatropine	337.54 (8)	143~~2.7~~ (10)	7~~.2~~ (4)	-	-
Apoatropine	-	-	4~~3.6~~ (4)	-	-
Scopoline	43~~2.8~~ (11)	21~~0.7~~ (16)	17~~.3~~ (6)	-	-
Tropine/physoperuvine	18~~.1~~ (6)	7~~6.6~~ (10)	17~~.1~~ (5)	3~~2.5~~ (12)	-
Pseudotropine	6~~5.5~~ (14)	2~~.4~~ (12)	3~~.4~~ (8)	-	-
Tropinone	-	-	10~~9.7~~ (7)	-	6~~.0~~ (1)

[a] Repeatability is given in brackets (n=3)

[b] 1 g/kg = 1g seeds per 1 kg of buckwheat

Figure

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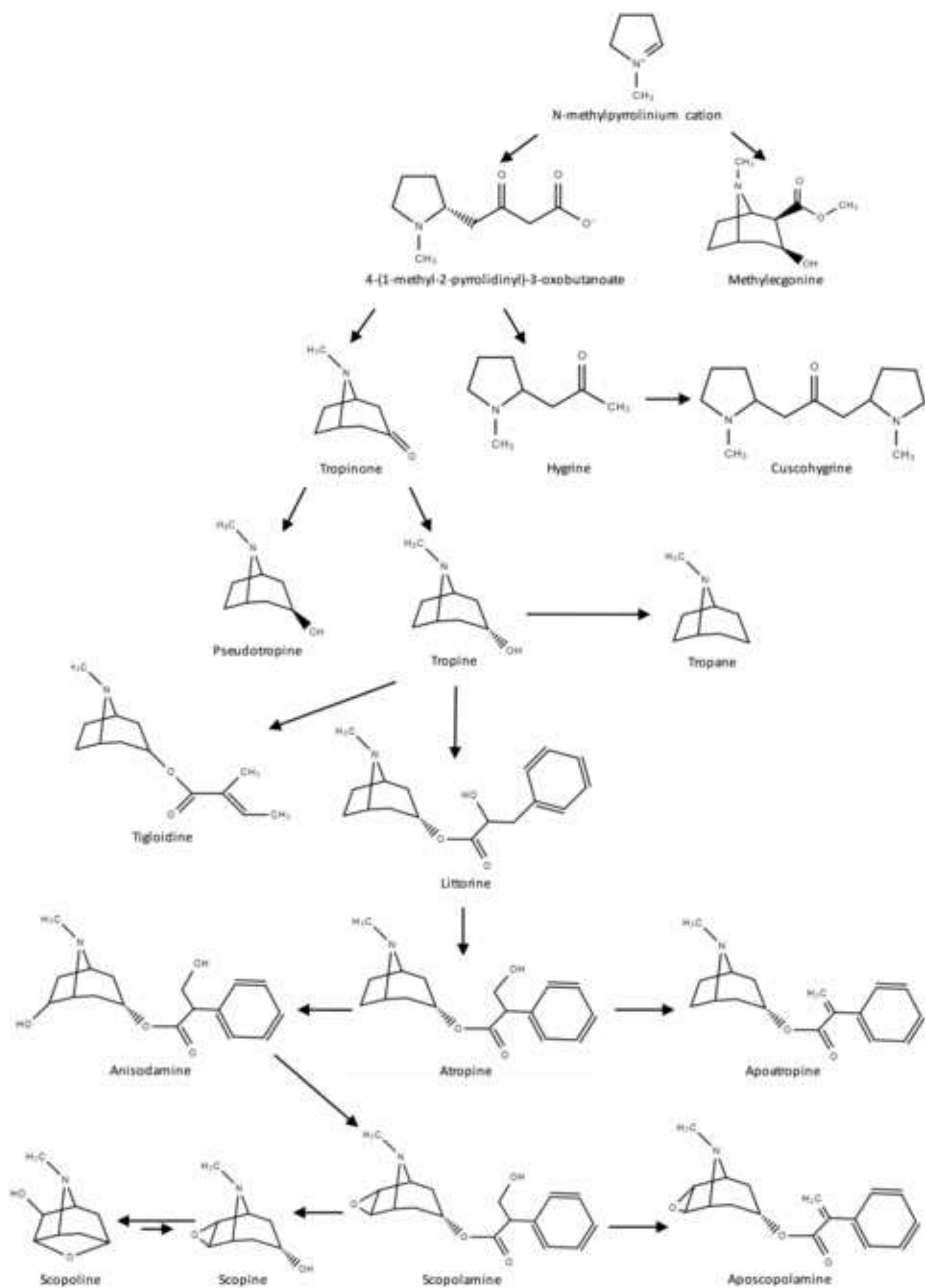

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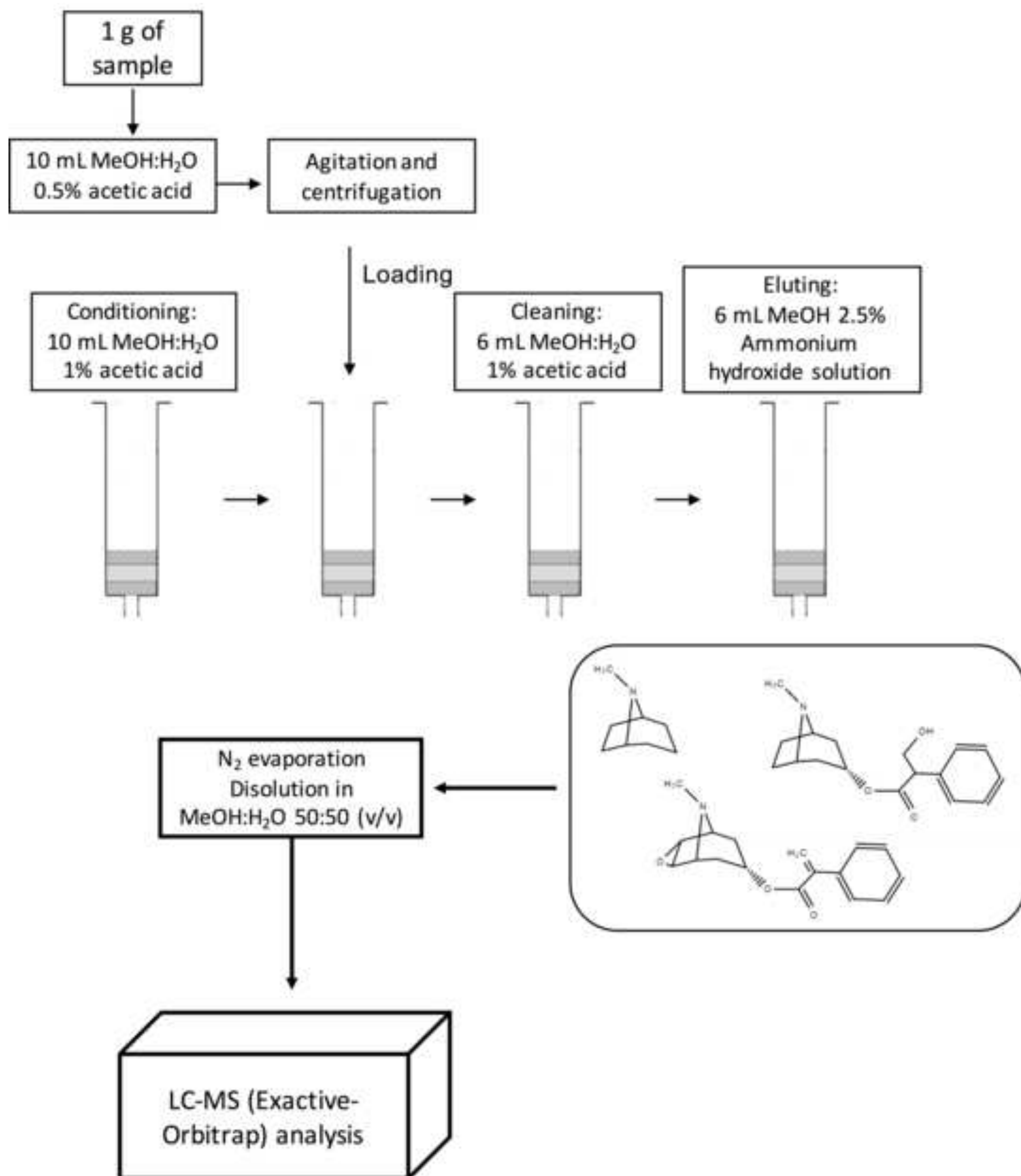

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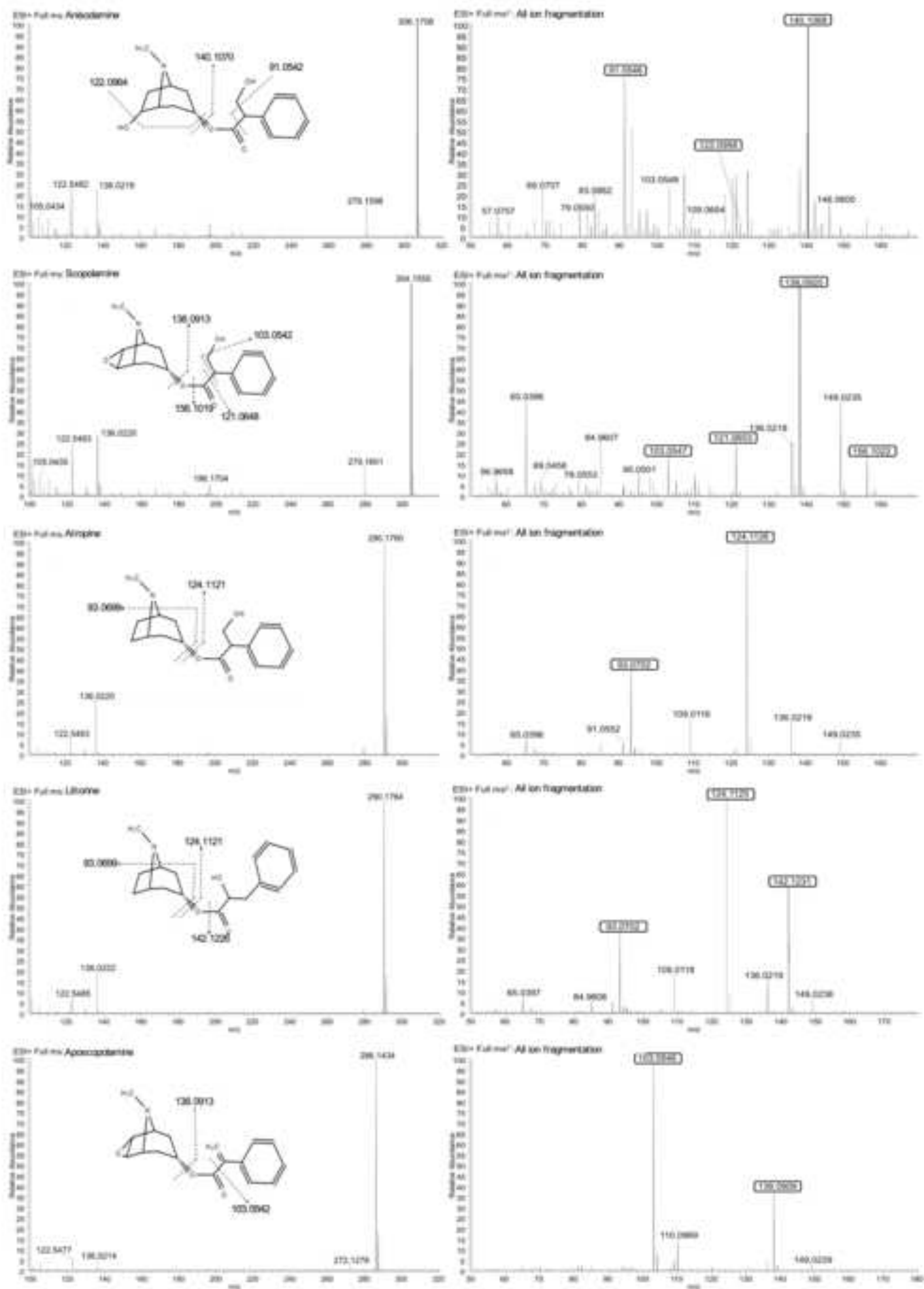

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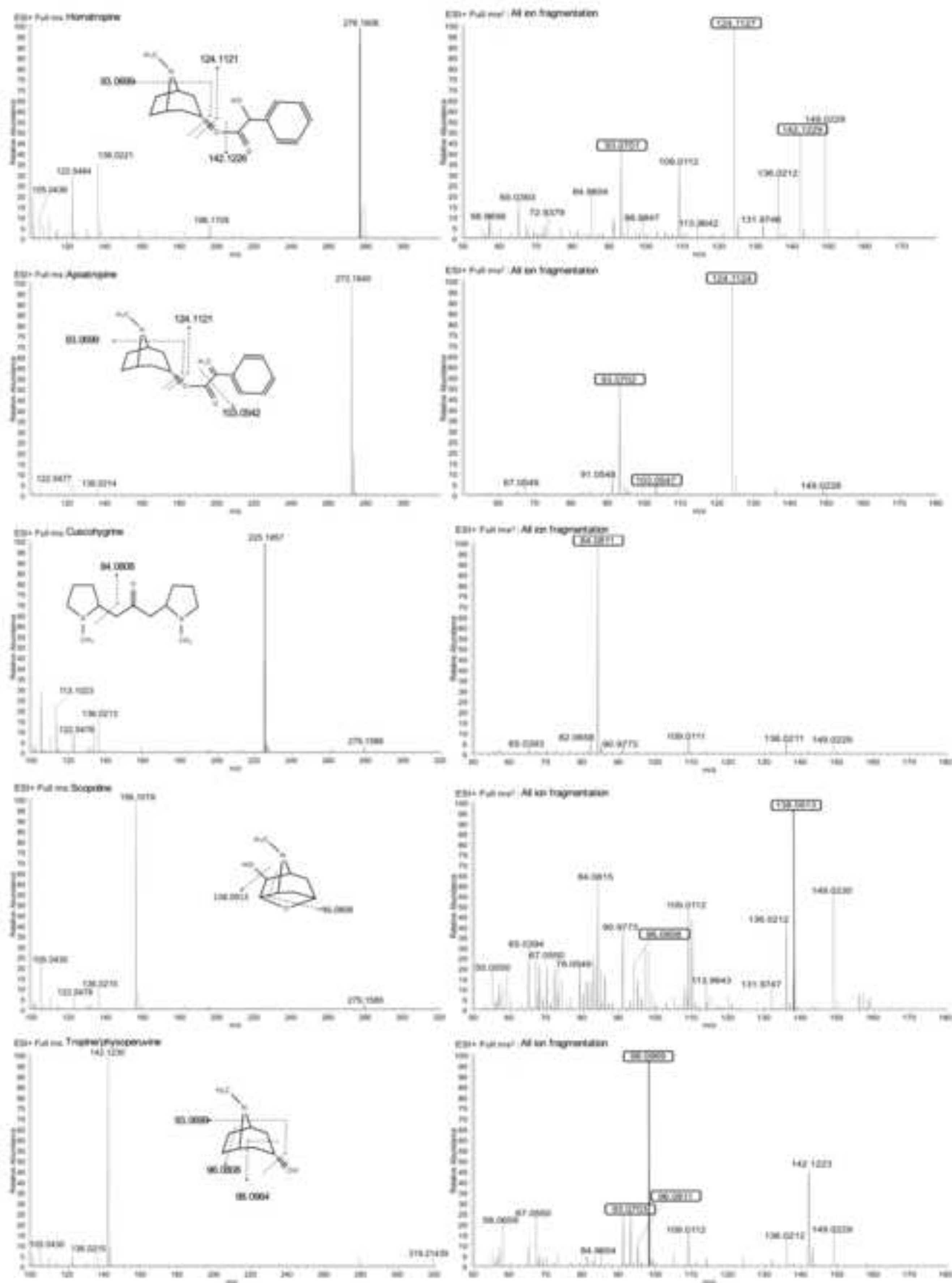

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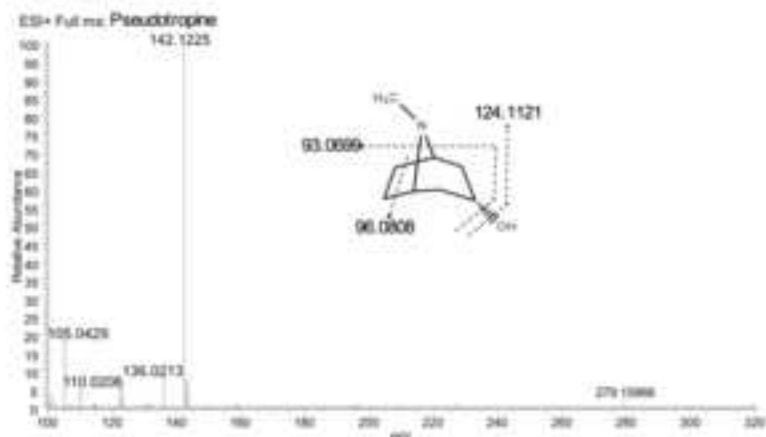

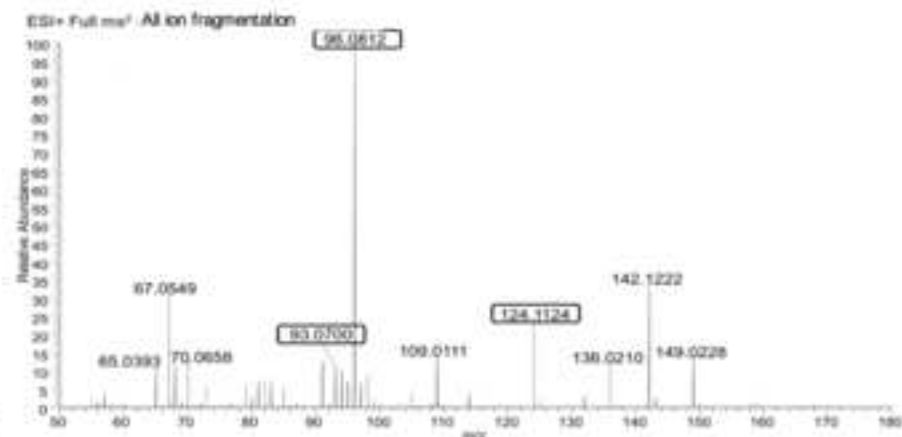

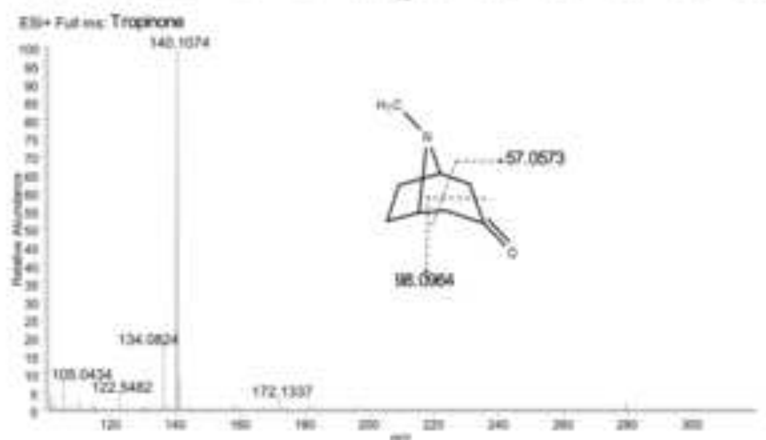

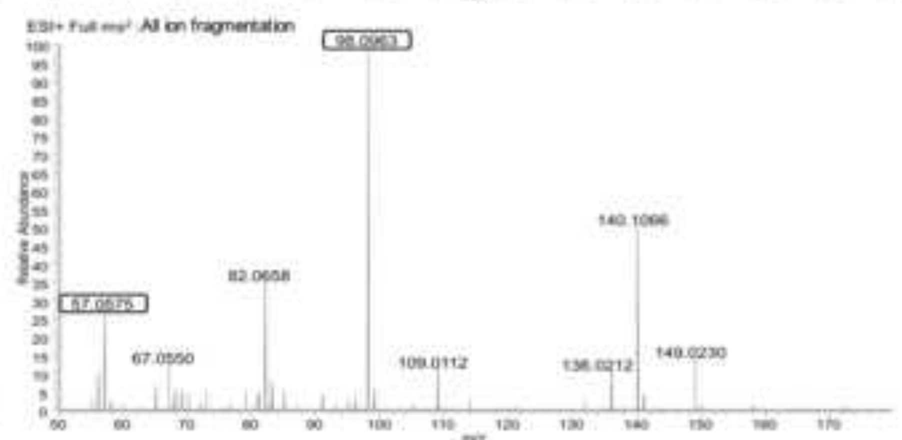

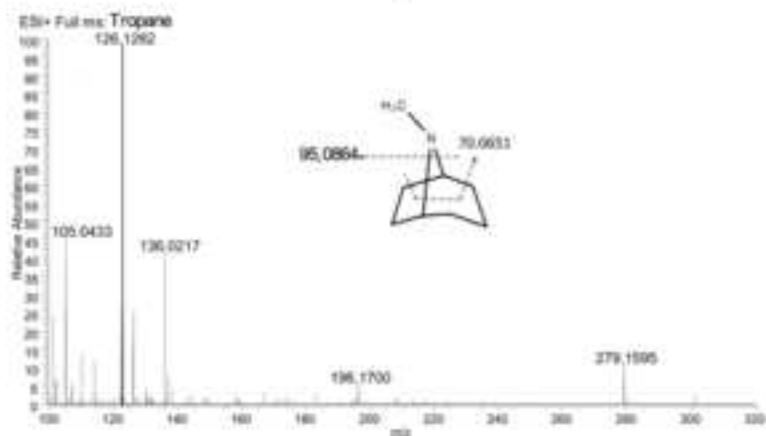

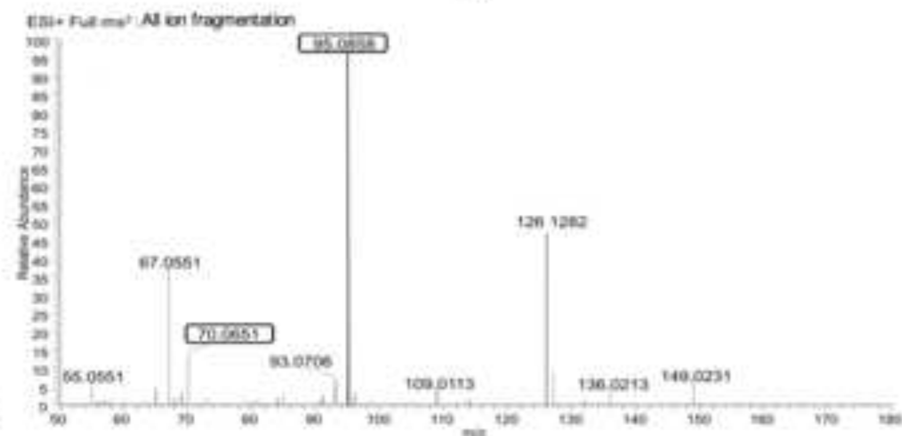

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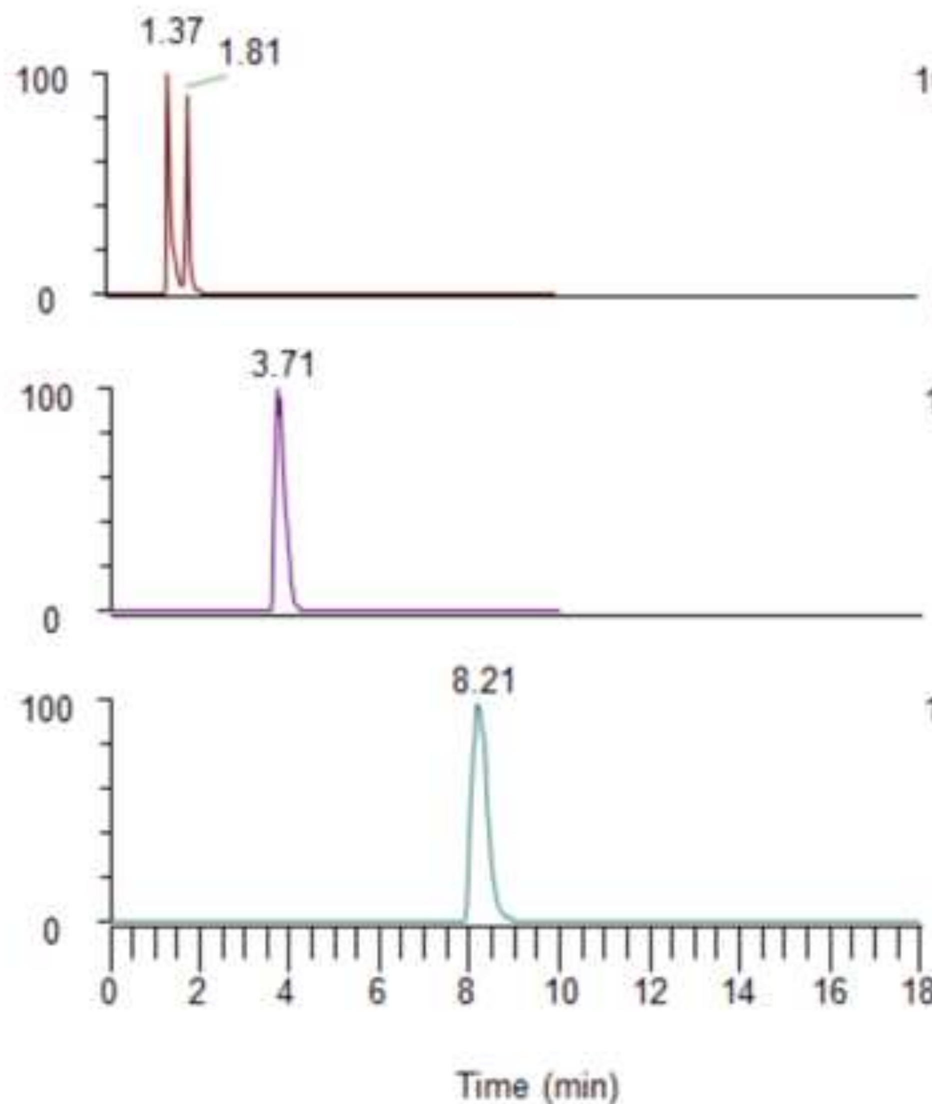

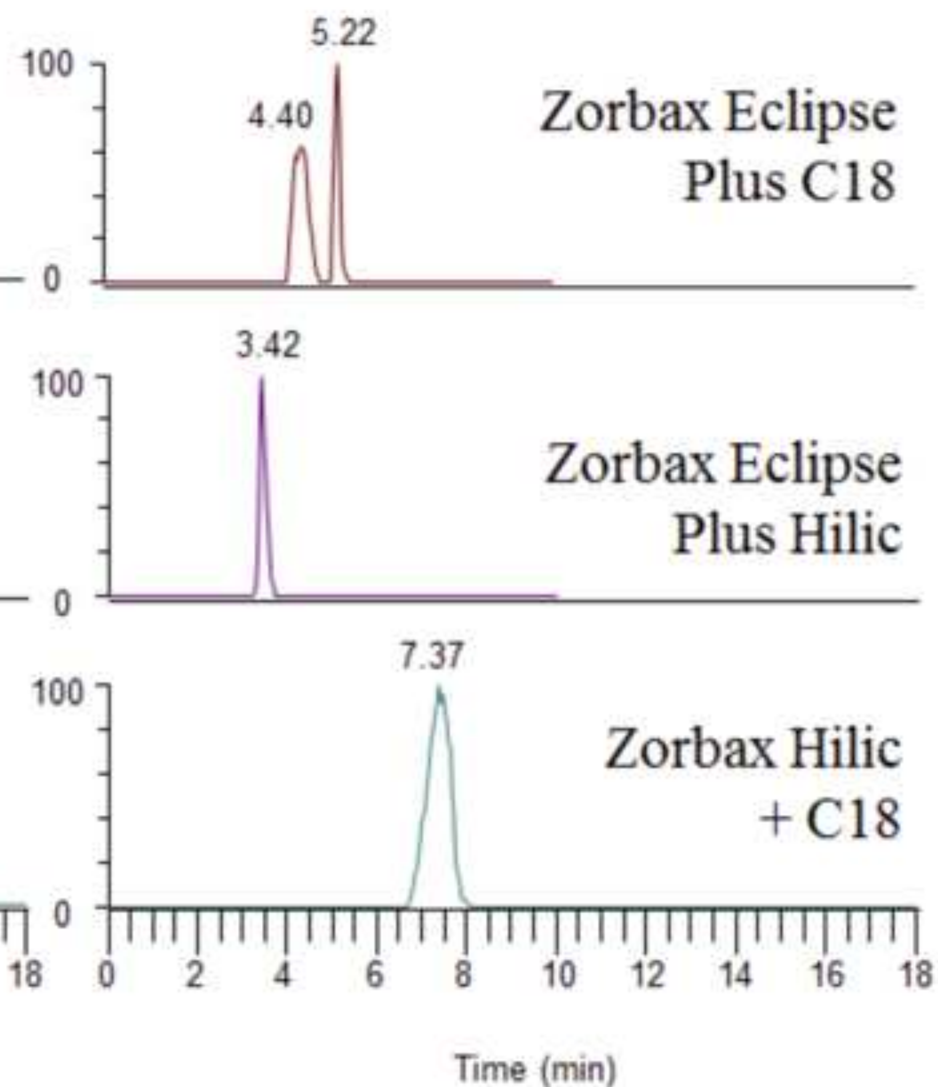

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